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Oxygen accelerates the accumulation of mutations during the senescence and immortalization of murine cells in culture

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Summary

Oxidative damage is a causal factor in aging and cancer, but it is still not clear how DNA damage, the cellular responses to such damage and its conversion to mutations by misrepair or misreplication contribute to these processes. Using transgenic mice carrying a *lacZ* mutation reporter, we have previously shown that mutations increase with age in most organs and tissues *in vivo*. It has also been previously shown that mouse cells respond to oxidative stress, typical of standard culture conditions, by undergoing cellular senescence. To understand better the consequences of oxidative stress, we cultured mouse embryo fibroblasts (MEFs) from *lacZ* mice under physiological oxygen tension (3%) or the high oxygen tension (20%) associated with standard culture, and determined the frequency and spectrum of mutations. Upon primary culture, the mutation frequency was found to increase approximately three-fold relative to the embryo. The majority of mutations were genome rearrangements. Subsequent culture in 20% oxygen resulted in senescence, followed by spontaneous immortalization. Immortalization was accompanied by an additional three-fold increase in mutations, most of which were G:C to T:A transversions, a signature mutation of oxidative DNA damage. In 3% oxygen, by contrast, MEFs did not senesce and the mutation frequency and spectrum remained similar to primary cultures. These findings demonstrate for the first time the impact of oxidative stress on the genomic integrity of murine cells during senescence and immortalization.

Key words: aging; cancer; DNA damage; genomic integrity; oxidative damage.

Introduction

Aging is a multifactorial process that results in a decline in the fitness of adult organisms, leading ultimately to death. Numerous theories have been proposed to explain the aging process. Among the most well known and accepted of these is the free radical theory, first proposed by Harman (1956). According to this theory, the essential aerobic metabolism that occurs in mammals and many other organisms produces reactive oxygen species (oxygen-free radicals or ROS), formed in the mitochondria as a by-product of oxidative phosphorylation to generate ATP. Virtually all cells that undergo aerobic metabolism possess antioxidant defence mechanisms that help neutralize the effects of ROS, which can attack many biologically important molecules, including DNA (Cooke *et al*., 2003). When ROS production exceeds the antioxidant defences, cells experience oxidative stress. Oxidative stress, in turn, can trigger the mutually exclusive processes of cell death or proliferative arrest, depending on the severity and tissue context. In many cases, oxidative stress causes cells to die by programmed cell death or apoptosis, and to arrest proliferation irreversibly by cellular senescence. Oxidative stress can also damage DNA, which, through erroneous repair or replication, can be converted into mutations. Mutations are now recognized as a major cause of cancer, and have also been proposed to contribute to aging (Vijg, 2000).

Since the development of transgenic mice harbouring chromosomally integrated bacterial mutation reporter genes, it has been possible to quantify and characterize the somatic mutations that accumulate in various mouse organs and tissues as the animals age and/or develop cancer (Gossen *et al*., 1989; Boerrigter *et al*., 1995; Vijg *et al*., 2002). Using these mice, we have previously shown that mutations accumulate with age in an organ- and tissue-specific manner (Dollé *et al*., 1997, 2000; Vijg *et al*., 1997). The origin of these age-related mutations is not known at present. However, it is tempting to speculate that they result from the accumulated effects of endogenous oxidative stress, to which these and all aerobic organisms are subject.

Mammalian tissues generally experience oxygen (O_2) concentrations ranging from 2 to 8% (Vaupel *et al*., 1989), well below

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the 20% O_2 present in air (atmospheric oxygen). Nonetheless, most mammalian cells are cultured in atmospheric oxygen. Parrinello *et al*. (2003) recently showed that primary mouse fibroblasts cultured in 20% O_2 accumulate oxidative DNA damage and undergo senescence as a result of severe oxidative stress, and that spontaneous immortalization involves an event that permits the cells to proliferate despite the damage. Primary human fibroblasts, by contrast, experience much less oxidative DNA damage in 20% oxygen (Parrinello *et al*., 2003). Instead, telomere dysfunction is an important, albeit in some cases not an exclusive, cause of senescence in human cells, which also rarely, if ever, spontaneously immortalize (Campisi, 2003). It is conceivable that mice have a higher rate of cancer and aging than humans, as a function of chronological age, at least in part because mouse cells are more susceptible to oxidative DNA damage than human cells.

To determine the molecular consequences (mutations) of oxidative damage to DNA during the proliferation of primary mouse cell cultures, we studied embryo fibroblasts from mice harbouring a silent bacterial *lacZ* mutation reporter gene by culturing them under ambient (20%) or *in vivo* (3%) oxygen tensions. The results indicate that cell culture conditions, including oxygen, greatly influence mutation accumulation and senescence in mouse embryonic fibroblasts.

Results

Spontaneous mutant frequencies *in vivo* and in culture

We have used mice that carry a *lacZ* transgene integrated as part of a plasmid at one or few chromosomal locations to obtain an estimate of the spontaneous mutation load of the somatic genome in various organs and tissues of aging mice (Dollé *et al*., 1997, 2000; Vijg & Dollé, 2002). Genomic instability owing to mutations has been implicated in loss of cellular proliferative homeostasis in culture and *in vivo* in mice and humans (Martin, 1979; Hasty *et al*., 2003). Murine cell cultures undergo replicative senescence and immortalization, which are thought to be important in aging and cancer, respectively, and do so much more readily than similarly cultured human cells (Campisi, 2003). However, very little is known about the mutation loads experienced by cells as they transition from primary culture through senescence and immortalization. We therefore used cells isolated from *lacZ* transgenic mice to follow the mutation frequency and spectrum as murine cells are taken from their natural environment *in vivo* into culture, and subsequently undergo senescence and immortalization.

Figure 1 illustrates the approach we followed to compare mutation frequencies in culture and *in vivo*. We isolated DNA from three types of cell population: mouse embryos, mouse embryonic fibroblasts (MEFs) in primary culture (first passage), and liver and small intestine of young adult mice (3 months of age). As a positive control for the mutation reporter assay, we also isolated DNA from early passage MEFs that were irradiated in culture with 5 J m^{-2} ultraviolet (UV) radiation, a well-characterized mutagen. We then separated and recovered the integrated mutation reporter plasmids from the isolated DNA, and scored the frequency of mutant plasmids relative to the total number of plasmids recovered, as previously described (Boerrigter *et al*., 1995; see Experimental procedures).

Relative to the *in vivo* situation (embryos and young adult liver and intestine), MEFs showed a two- to three-fold higher mutation frequency, which was evident even at the first passage (Fig. 2A). This increase in mutation frequency was not significantly influenced by the oxygen tension used for cell culture because it was observed irrespective of whether the cells during their first passage were kept at 3% O_2 or at 20% (data not shown). It should be noted that for cells kept at 3% O_2, exposure to atmospheric oxygen nevertheless occurred during isolation of the cells from the embryo and during trypsinization when splitting the culture.

To understand the nature of the mutations that occurred *in vivo* and in culture, we obtained an initial characterization of the mutant plasmids rescued from the mouse embryos, adult tissues and first passage MEFs. For this purpose, we subdivided the mutations into those that did not change the size of the plasmid insert (no-change mutations) and those that altered the size by 50 bp or more (size-change mutations). No-change mutations are indicative of point mutations, small insertions and small deletions, whereas size-change mutations are indicative of large genome rearrangements or deletions (Dollé *et al*., 1997, 2000). By these criteria, the increase in mutation frequency from embryo to primary cells did not appear to alter the mutation spectrum, which consisted predominantly of size-change mutations. That is, size-change mutations were about two-fold more prevalent than no-change mutations in both embryos and primary cells (Fig. 2A). Interestingly, size-change mutations were less prevalent in the adult tissues, where they comprised roughly 25% (small intestine) and 45% (liver) of the total mutations detected.

As expected, our positive control, treated with 5 J m^{-2} UV, a relatively modest UV dose, increased the mutation frequency of MEFs approximately 14-fold, and most of these mutations were of the no-change class, i.e. point mutations, which is typical for UV (Fig. 2B).

Mutant frequencies and spectra of MEFs cultured in high or low oxygen

Under standard culture conditions, which include 20% O_2, MEFs undergo replicative senescence, which can be completely bypassed by culturing the cells under more physiological oxygen tensions, such as 3% O_2 (Parrinello *et al*., 2003). We therefore investigated whether mutations accumulated as MEFs underwent senescence, i.e. at 20% O_2. The results of two typical experiments using MEFs from two different embryos are shown in Fig. 3, with the estimated time points of senescence or immortalization indicated by arrows. As expected (Todaro & Green, 1963; Parrinello *et al*., 2003), MEFs cultured in 20% O_2 exhibited retarded growth owing to senescence, but no sign of

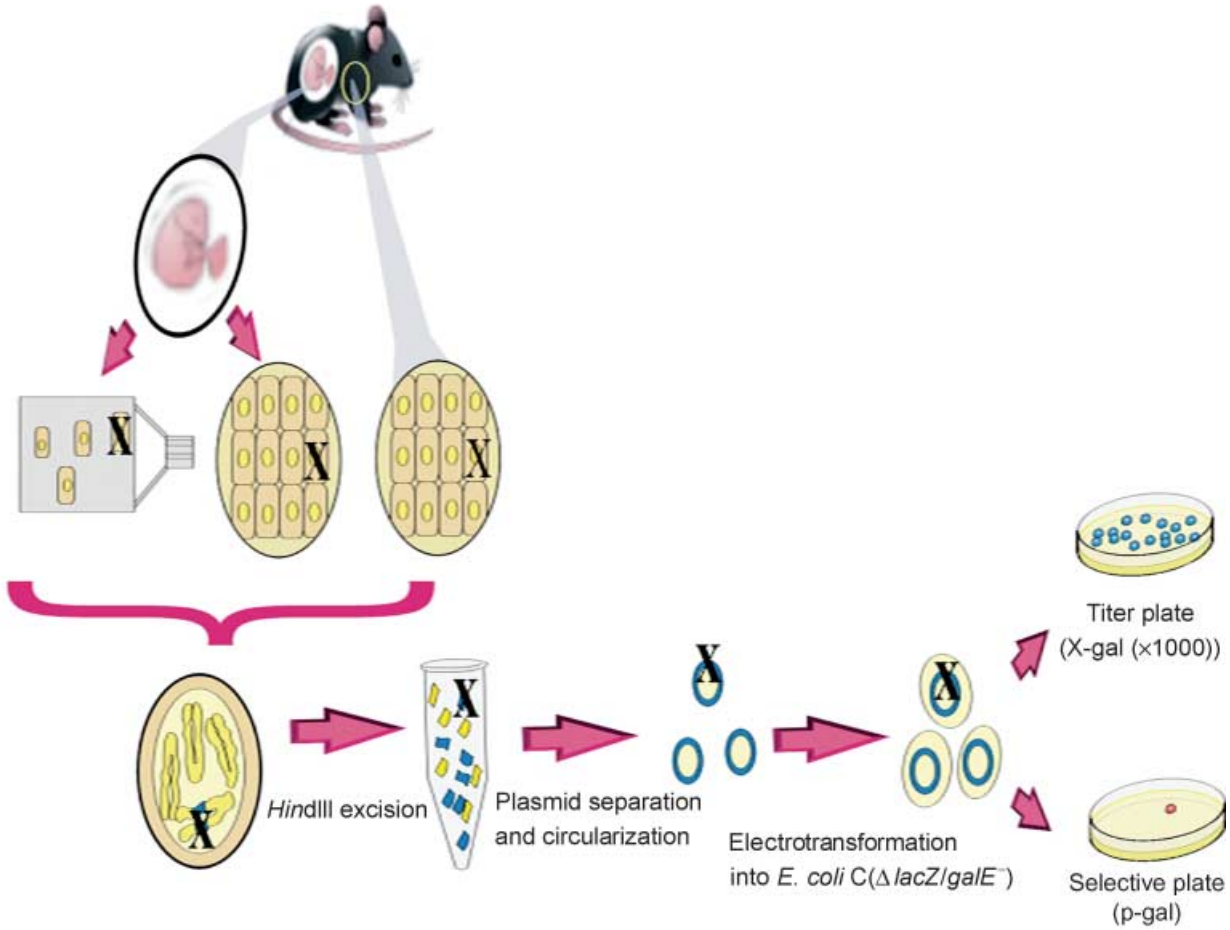

Fig. 1 Strategy for determining mutation frequency and spectra using transgenic mice carrying integrated *lacZ* mutation reporter plasmids. A three-fold comparison was made, i.e. between young adult tissue, embryonic tissue and first passage cells isolated from the embryo. After DNA extraction, plasmids (blue areas), both wild-type and those containing a mutation (X), were excised from genomic DNA and analysed for the frequency and type of mutations in the different cell populations, as described in Experimental procedures.

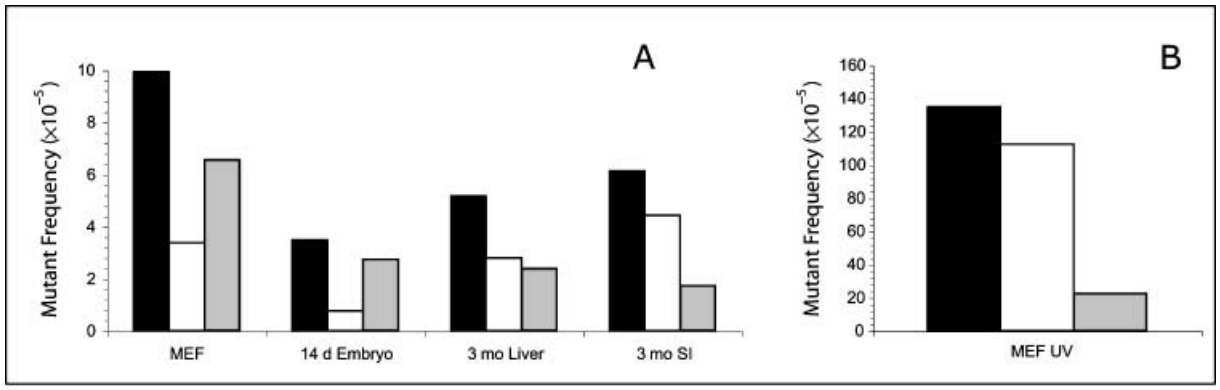

Fig. 2 (A) Mutation frequencies at the *lacZ* locus in actively proliferating first passage MEFs, 14-day-old embryo carcasses, and the liver and small intestine of 3-month-old mice. (B) Mutation frequencies of early passage MEFs irradiated with 5 J m^{-2} UV. Reporter plasmids were rescued and characterized as described in Experimental procedures. The solid black bars show the total mutant frequency, the white bars show the frequency of no-change mutations (point mutations, small insertions or small deletions), and the grey bars show the frequency of size-change mutations (genomic rearrangements and large deletions).

senescence was observed when the cells were cultured in 3% O_2 (Fig. 3A,B). In both 20% and 3% O_2, the mutation frequency remained relatively constant at approximately the level observed in first passage MEFs until about the time the cells in 20% O_2 reached senescence (Fig. 3C,D; compare early time points with Fig. 2A). Thereafter, the mutation frequency remained constant, but only in the 3% O_2 cultures (Fig. 3C,D). By contrast, as immortal variants overgrew the 20% O_2 cultures (Fig. 3A,B),

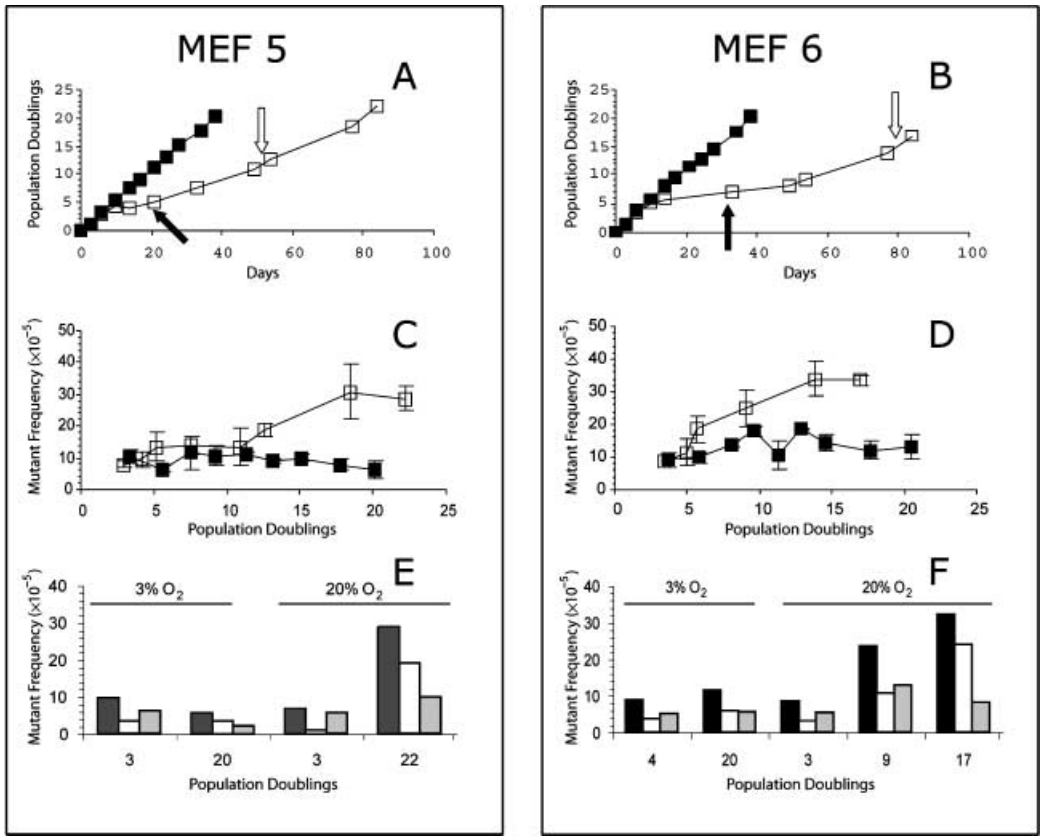

Fig. 3 (A,B) Growth of MEFs from two different embryos (MEF5 and MEF6), at 3% (solid symbols) or 20% (open symbols) oxygen. The approximate point at which the cells underwent senescence is represented by solid arrows whereas open arrows represent the point of immortalization. Population doubling levels were calculated on the basis of the cell counts when passaging the cultures (see Experimental procedures). (C,D) *lacZ* mutation frequencies of MEF5 and MEF6 at 3% and 20% oxygen as a function of population doubling level. (E,F) Frequencies of point mutations and size-change mutations of MEF5 and MEF6 at 3% and 20% oxygen at different population doubling levels. Solid black bars show the total mutant frequency, white bars show the frequency of no-change mutations (point mutations, small additions or small deletions) and grey bars show the frequency of size-change mutations (genomic rearrangements and large deletions).

the mutation frequency increased approximately three-fold (Fig. 3C,D). This elevated mutation frequency appeared to stabilize after the immortalized cultures had completed 15–20 population doublings (PDs). Of note, the increase in mutation frequency at high oxygen as compared to cells at 3% O_2 was also observed when the mutation frequency was plotted as a function of time in culture rather than population doubling levels (data not shown).

To determine the kinetics of mutation accumulation more accurately, we measured the mutation frequencies in a total of six MEF cultures and plotted them against the number of PDs completed (Fig. 4). The combined results show that the mutation frequency gradually rises in 20% O_2, beginning on average at about PD5, which is when these cultures typically senesce and immortal variants begin to arise (Fig. 3A,B). These findings suggest that immortalization rather than senescence is accompanied by a substantial increase in mutation frequency.

To determine the types of mutations that accumulate in 20% and 3% O_2, the reporter plasmids were rescued and scored for no-change or size-change mutations, as described for Fig. 2(A,B). MEFs cultured in 3% O_2 did not accumulate mutations over approximately 20 PD, and the fraction of no-change and size-change mutations remained relatively constant (Fig. 3E,F). However, cells cultured for > 5 PD in 20% O_2 showed a striking (eight- to 19-fold) increase in no-change mutations, with very little increase in size-change mutations. These results indicate that immortalization in 20% O_2 is accompanied predominantly by an increase in point mutations.

To determine more precisely the nature of the mutations that accumulate in MEFs at 20% O_2, we sequenced 20 no-change mutant plasmids rescued from MEFs grown for approximately 17–22 PD in 20% O_2, and 20 no-change mutant plasmids obtained from MEFs grown for 20 PD in 3% O_2. This was done for two different MEF cultures, each derived from an independent embryo. Regardless of the oxygen tension under which the cells were cultured, most (> 95%) no-change mutations were base pair substitutions; very few (< 5%) were small insertions or deletions (Fig. 5). Moreover, most of these mutations were found only once, indicating that they were not due to one or very few mutations that arose early and were subsequently propagated in the population (i.e. 'jackpot mutations'). Interestingly, nearly half of the mutations in cells cultured in 3% O_2 were G:C to A:T transitions, whereas the majority of mutations found in cells cultured in 20% O_2

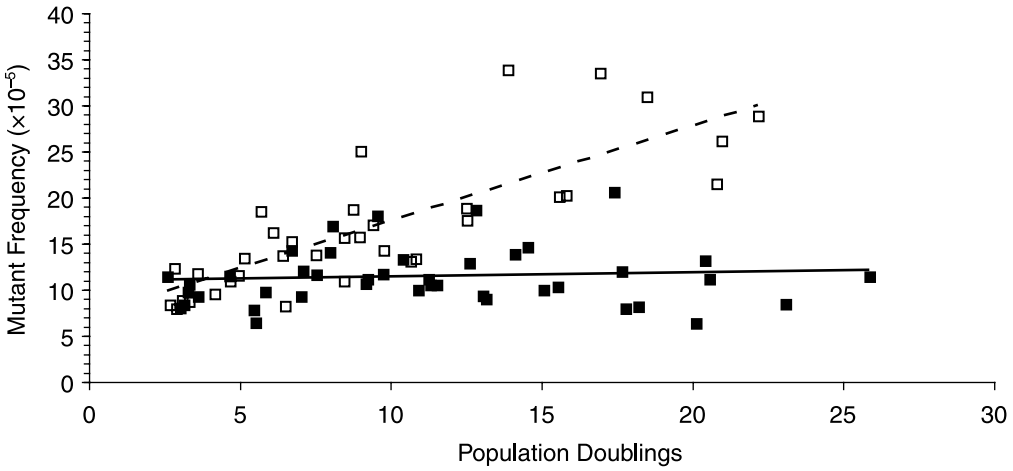

Fig. 4 Average mutant frequencies of six independent MEF cultures at 3% (solid symbols, solid line) or 20% (open symbols, broken line) oxygen as a function of population doubling level.

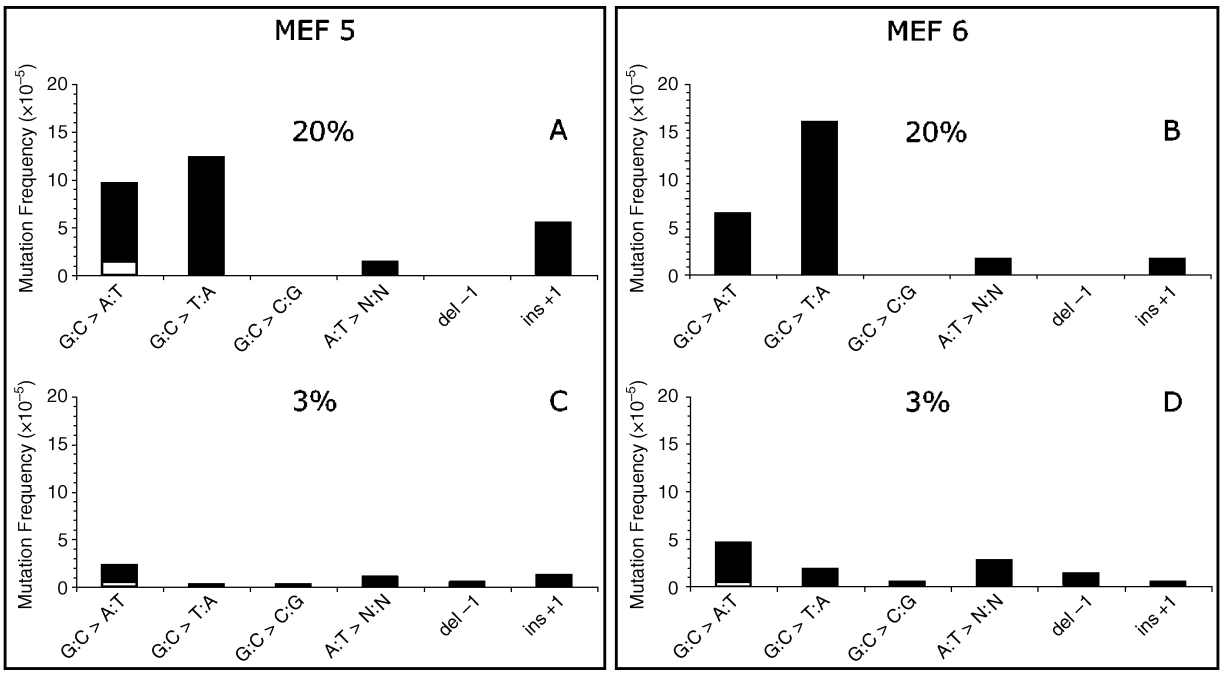

Fig. 5 Point mutational spectra of MEFs cultured in 20% or 3% oxygen. The MEF cultures described in Fig. 3 were harvested at late (PD17, 22) passages, and recovered plasmids were sequenced as described in Experimental procedures. The white areas in the G:C to A:T bars indicate the fraction of these mutations that occurred at CpG sites.

were the result of G:C to T:A transversions, the signature mutation of 8-oxo-guanine (8-oxo-7,8-dihydroguanine) (Cunningham, 1997). Of the G:C to A:T transitions in cells at 3% oxygen, only a small percentage had occurred at CpG sites, indicating that very few of these mutations had been caused by deamination of 5-methylcytosine. Because the *lacZ*-plasmid cluster is highly methylated *in vivo*, the lack of such mutations is consistent with the finding that passage in culture often results in general demethylation of genomic DNA (Matsumura *et al*., 1989).

Discussion

Using a chromosomally integrated *lacZ* reporter gene that can be recovered into *E. coli* for quantification and characterization of the mutation load, we measured the genomic integrity of cells from their transfer from the mouse embryo into culture and, during their growth, senescence and immortalization in high or low oxygen. Soon after placement in culture, regardless of the oxygen level, mutations increased two- to three-fold. Thus, there was a nearly immediate increase in mutation

frequency during the first passage in culture. Thereafter, the mutation frequency remained stable for > 20 PD when MEFs were cultured in low oxygen, which prevents replicative senescence (Parrinello *et al.*, 2003). The mutation frequency also remained stable in cells cultured in high oxygen, but only until senescence. Cells in high oxygen showed a further gradual increase, concomitant with immortalization, amounting to an additional three-fold rise that stabilized after 15–20 PD. This second increase in mutation frequency was almost certainly due to oxidative DNA damage, as indicated by the presence of 8-oxo-guanine signature mutations, G:C to T:A transversions, and the absence of such an increase, with virtually no G:C to T:A mutations, in low oxygen.

What might be the cause of the first increase in mutation frequency, which occurs so rapidly when MEFs are placed in culture? First, it is possible that during embryo dissection and dispersion, which occurred in 20% O_2, oxidative DNA lesions were introduced, even if the subsequent first passage occurred in 3% O_2. Because the developing embryo and first passage cells in culture have similar rapid cell proliferation kinetics, oxidative damage might lead to a mutation spectrum dominated by genome rearrangements. Indeed, oxygen does induce predominantly genome rearrangements in rapidly proliferating cells (Gille *et al.*, 1994) and oxidative stress has been found to be associated with genomic instability (Barnes, 2002). Alternatively, the possibility cannot be ruled out that other factors associated with the isolation of cells from the tissue and their adaptation to culture are responsible for this firmly established increase in mutation frequency associated with the transition from the *in vivo* to the *in vitro* situation.

If the first increase in mutation frequency results predominantly in genome rearrangements, why then does the second increase in mutation frequency, which is clearly caused by high oxygen, result predominantly in point mutations, i.e. mainly G:C to T:A transversions? Although oxygen may predominantly induce genome rearrangements, the high toxicity of this type of mutation, with each individual event affecting large regions of the genome, may not permit a significant further increase above the one immediately obtained upon culture. Hence, a further increase in mutation frequency as a consequence of continuous culture at high oxygen will only be revealed by an increase in point mutations, of which G:C to T:A transversions have been conclusively demonstrated to be associated with the main oxidative lesion 8-oxoguanine (Sekiguchi & Tsuzuki, 2002). The transition G:C to A:T mutations observed at low oxygen may reflect other causes of spontaneous mutations in rapidly dividing cells, such as replication errors *per se* or weakly mutagenic factors in the culture medium.

Of interest is the potential contribution of the increased mutation frequency in facilitating the cells to escape from senescence through immortalization, which is almost certainly a consequence of mutations. The high spontaneous mutation frequency of the cultured cells would also increase the chance of mutational inactivation of a genome maintenance pathway, which would accelerate the mutation frequency further (Loeb, 1998), and thus the likelihood of inactivating genes that control the senescence response. In this respect, it may be more likely that a mutator phenotype develops from inactivating mutations in DNA mismatch repair or excision repair pathways, rather than pathways that repair the highly toxic DNA double-strand breaks. Such mutants, i.e. cells with a very high frequency of point mutations, would quickly predominate the cell culture. Indeed, the possibility cannot be excluded that the second increase in mutation frequency may reflect a shift in the cell population structure rather than an increase in point mutations in all cells.

Our findings are consistent with DNA damage rather than mutations causing the senescence of MEFs cultured in 20% O_2, but it is also possible that the genomic rearrangements found to accumulate almost immediately upon primary culture could cause a state of stress and eventually evoke a senescence response when these cells are continuously exposed to high oxygen. This possibility is consistent with the finding that clastogens, such as mitomycin C and ionizing radiation, cause a senescence response in human cells (reviewed in Campisi, 2003). Genome instability in the form of large random rearrangements would be an attractive explanation for the phenomenon, especially for mouse cells, in which telomere shortening does not play a role in senescence.

It has been proposed that basic mechanisms responsible for aging in intact organisms can also occur in cultured cells. Indeed, replicative senescence was first formally described for human cell cultures, and was interpreted as aging at the cellular level (Hayflick & Moorhead, 1961). It is now thought that telomere shortening, as a result of DNA replication in the absence of telomerase, is the primary cause for the replicative senescence of human cells (Campisi, 2003). Mouse cells, however, constitutively express telomerase and most likely undergo replicative senescence owing to the oxidative stress of standard culture conditions, to which mouse cells appear much more sensitive than human cells (Parrinello *et al.*, 2003). Nonetheless, human cells also senesce in response to oxidative stress, which in turn can cause premature telomere shortening (von Zglinicki *et al.*, 1995). Moreover, human cells cultured in 2–3% O_2 undergo 20–50% more PDs (Packer & Fuehr, 1977; Balin *et al.*, 1984; Shigenaga & Ames, 1991; Chen *et al.*, 1995; Saito *et al.*, 1995). By contrast, mouse cells do not senesce at all in 3% O_2 (Parrinello *et al.*, 2003). Thus, the difference in oxygen sensitivity between mouse and human cells in culture is one of degree. Our findings that mouse cells accumulate mutations even after brief oxidative stress may explain their propensity to escape senescence and immortalize in culture, a process that appears to be important in the development of cancer (Meek *et al.*, 1977; Campisi, 2003). Thus, our results may help explain the high incidence of cancer that is seen in mice. It remains to be seen whether the mutations induced by oxidative stress play a role in their longevity.

Experimental procedures

Cell isolation and culture

pUR-288-lacZ transgenic mice are homozygous for the bacterial *lacZ* gene at two loci (on chromosomes 3 and 4) (Dollé *et al*., 1997). Day 14 embryos from these mice were used to isolate fibroblasts (MEFs) as described (Parrinello *et al*., 2003). Briefly, the placental membranes, amniotic sac, head and primordial blood organs were removed and the remaining carcass was washed and minced in 2 mL phosphate-buffered saline (PBS) using a syringe and 18-gauge needle, passed through a 100-μm strainer to remove large fragments, and placed in a 25-cm^2 flask containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin and buffered with bicarbonate. We incubated the tissue suspension in 10% CO_2 and 3% O_2, using an incubator equipped with an O_2 sensor and regulator and a N_2 gas source. After 3 days, cells were transferred to a 75-cm^2 flask and cultured until 90% confluent. This enriched fibroblast population was then frozen, or 10^6 cells were subcultured in 150-cm^2 flasks and considered passage 1 and PD 0. At this point, the cells were maintained in 3% O_2 or switched to 20% O_2. Trypsinization and passaging were performed in atmospheric (20%) oxygen. Each MEF culture used for each experiment was derived from a single embryo. Cells were passaged at 10^6 per 150 cm^2 when they reached ~80% confluency. At each passage, cell number was determined and PD levels calculated as described (Rubio *et al*., 2002). In general, three 150-cm^2 flasks were passaged per condition and cells were either subcultured for the next passage or collected by centrifugation and frozen for analyses.

DNA isolation

DNA was isolated from embryo carcasses or adult tissues as described (Dollé *et al*., 1996). Two to three 150-cm^2 flasks of cells were used for DNA isolation from cell cultures. Cultured cells were trypsinized and collected by centrifugation at 4 °C. The cell pellet was washed with PBS and stored at −80 °C. Frozen pellets were suspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% SDS, 0.5 mg mL^{-1} Proteinase K, 120 μg mL^{-1} RNase A), incubated overnight at 50 °C with agitation, extracted twice with phenol–chloroform–isoamyl alcohol (25 : 24 : 1), followed by addition of 1/5 volume of 8 M potassium acetate and extraction with 1 volume of chloroform. The DNA was precipitated by addition of ethanol, washed with 70% ethanol and solubilized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. DNA concentration was determined by measuring the OD at 260 nm.

Mutation frequency determination

Mutation frequencies were determined as described (Dollé *et al*., 1996). Briefly, 20 μg of genomic DNA was digested with *Hin*dIII and the plasmids thus excised were separated from the remaining mouse genomic DNA using magnetic beads precoated with lacZ/lacI fusion protein to bind the operator sequence of the *lacZ* gene. After washing, plasmid DNA was eluted from the beads using isopropylthio-β-galactoside (IPTG), circularized with T4 DNA ligase and electroporated into *E. coli* C (*ΔlacZ*, *galE*$^-$) host cells. To determine the total number of transformants, 0.1% of the transformed bacterial cells were plated in agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The remainder was plated in 0.3% phenyl-galactoside (p-gal) to select for cells harbouring plasmids with *lacZ* mutations (only the *lacZ* mutant *E. coli* C *galE*$^-$ cells can grow in this medium). Mutation frequencies were calculated as the number of mutant colonies divided by the number of recovered transformants. Each mutation frequency determination is based on at least 300 000 recovered plasmids per culture.

Mutation classification and characterization

Mutant colonies from selective plates were transferred to 96-well round-bottomed plates and cultured overnight at 37 °C in 150 μL LB medium, 25 μg mL^{-1} kanamycin and 75 μg mL^{-1} ampicillin. One microlitre was plated onto X-gal plates to screen for galactose-insensitive host cells and this background was subtracted (Dollé *et al*., 1996). One microlitre was added to a mix containing 12.5 μL HotStarTaq (Qiagen) and 12.5 μM each of 4932-F (5′-TGGAGCGAACGACCTACACCGA-3′) and 3829-R (5′-ATAGTGTATGCGGCGACCGAGT) primers, and the DNA amplified by the polymerase chain reaction (PCR) using an initial 10 min at 95 °C followed by 35 cycles of 20 s at 95 °C and 8 min at 68 °C, and a final extension of 10 min at 68 °C. The resulting PCR product was digested with AvaI and size separated on a 1% agarose gel to classify mutations as no-change or size-change plasmids. The mutant spectra were adjusted for *Hin*dIII star-activity mutants as described (Dollé *et al*., 1999). For each sample at least 48 mutants were characterized.

lacZ genes of selected plasmids were sequenced entirely (from base 1 to base 3309, with reference to the pUR288 plasmid sequence available in GenBank under the name SYNPUR288V) using the following primers (5′→3′): pUR0366-F (CAGTTGCGCAGCCTGAATGG), pUR0794-F (TGACGGCAGTTATCTGGAAG), pUR1234-F (ATGGTCTGCTGCTGCTGAAC), pUR1671F (CAGTATGAAGGCGGCGGAGC), pUR2113-F (CCGGGCAAACCATCGAAGTG), pUR2552-F (GCCGCTGCGCGATCAGTTCA), pUR5268-F (CCGCTTTGAGTGAGCTGAT) and pUR3367-R (GCGTATCACGAGGCCCTTTC). Sequencing data were analysed using Sequencher (Gene Codes Corporation, MI, USA).

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